REMARKS

The Present Invention

The present invention is directed to an improved method for the coamplification of two or more target nucleic acids having different sequence compositions in a PCR reaction mixture. The improvement is directed to the use of a hot start DNA polymerase in combination with a polymeric volume exclusion agent, wherein the polymerase has been chemically reversibly inactivated and wherein the enzyme is reactivated at temperatures above 50°C. The nucleic acids are present in the reaction mixture at the start of the reaction at comparable copy numbers wherein any one population of target nucleic acid is not present at more than a 10-fold difference from any other target nucleic acid population in the reaction mixture. The improved method is particularly suited for multiplex PCR reactions that include two or more target nucleic acids and may include multiple sets of primers of different specificity in a single reaction mixture.

A hot start DNA polymerase which has been chemically-reversibly inactivated, i.e., "chemically-modified" is particularly advantageous over reversibly inactivating the enzyme by reaction with an anti-polymerase antibody. In a PCR reaction that includes an antibody-inactivated hot start polymerase, all the antibody is released, i.e., destroyed, from the enzyme in the first nucleic acid denaturation (typically 95°C) step of the first PCR cycle, which reduces the efficiency of the amplification reaction in subsequent cycles. The Examiner makes note of this in the present Office Action in the discussion of Backus. According to the Examiner,

> "Backus teaches a method for the coamplification of two or more target nucleic acids . . . comprising the sequential steps of: . . . wherein said hot start DNA polymerase is activated at T1 (col. 7, lines 11-19, where the inclusion of a polymerase and the **inclusion of an antibody specific to the DNA polymerase** which inhibits enzymatic activity below about 50°C and **which is inactivated at higher temperatures**) . . . " (see, Office Action, paragraph bridging pages 6 and 7.) (emphasis added)

In contrast, a chemically-modified hot start polymerase gradually recovers enzyme activity throughout the course of the entire multicycle reaction. In other words, all the chemical adducts do not dissociate from the polymerase during the first high temperature activation step of the first cycle; the inactivating effects of the chemical treatment of the enzyme are reversed in stages throughout the course of the entire reaction. This significantly improves the efficiency of amplification, particularly in later cycles of the reaction, by ensuring the availability of "fresh" or active polymerase in the late stage reaction cycles.

Applicants have amended independent Claims 1, 2, and 4 to recite this improvement. Specifically, Claims 1, 2, and 4 have been amended to recite that the method of the present invention includes a chemically-modified (i.e., reversibly-inactivated by chemical reaction) hot start DNA polymerase that is reactivated after incubation at temperatures above 50°C, in combination with from 1 to 20 weight % of a nonionic polymeric volume exclusion agent in at least one of the primary amplification cycles. Support for this amendment may be found at page 8, lines 16-18, and in the preamble of original Claims 1-4. Claim 3 has been canceled. In addition, Applicants have added new Claim 25 directed to a hot start DNA polymerase chemically-modified by reaction with an aldehyde. Support for this amendment may be found in the Examples section of the specification beginning on page 26. Hot start DNA polymerases which have been chemically-modified, for example, by reaction with an aldehyde are described in U.S. Pat. No. 6,183,998 entitled "Method For Reversible Modification Of Thermostable Enzymes", which is incorporated by reference into the present specification (see, page 27, line 9). No new matter has been added by the claim amendments

Entry of the amendments and allowance of Claims 1, 2, 4-16, 23-25, are respectfully requested.

35 U.S.C. §103(a)

The Examiner has rejected Claims 12-15 under 35 U.S.C. §103 as being unpatentable over Backus et al., U.S. Pat. No. 5,705,366[1], in view of Bustin, *Journal of Molecular Endocrinology*, 25: 169-193 (2000) as applied to claims 1-11 and 16 and further in view of Reed et al., U.S. Pat. No. 5,459,038 ("Reed") and Demke et al., *Biotechniques*, 12(3): 332-334 (1992) ("Demke").

According to the Examiner, with respect to Backus,

> "Backus discloses a method of amplification of multiple target nucleic acids in the presence of a nonionic, polymeric volume exclusion agent." (See, Office Action, page 3.)

With respect to Bustin, the Examiner cites the passage,

> "The accepted method for minimizing these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an

[1] Applicants note that the Examiner cites U.S. Pat. No. 5,703,366 throughout the Office Action. However, U.S. Pat. No. 5,703,366 is issued to Sting et al. and is entitled "Optical Sensing With Crystal Assembly Sensing Tip".

> internal reference against which other RNA values can be normalized. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. In addition, an exogenous control should also be expressed at roughly the same level as the RNA under study." (See, Office Action, page 5.)

As stated above, Applicants have amended independent Claims 1, 2, and 4 to further distinguish the present invention. In particular, the claims have been amended to recite that the invention is directed to the coamplification of two or more target nucleic acids in a reaction mixture, the improvement being the inclusion in the reaction mixture of a chemically-modified hot start DNA polymerase that is activated after incubation at temperatures above 50°C, in combination with a polymeric volume exclusion agent wherein the two or more target nucleic acids are present in comparable copy numbers where there is no more than a 10-fold difference between any one target nucleic acid and any other target nucleic acid in the starting reaction mixture. As demonstrated in the Examples section of the specification, the method is particularly suited to multiplex PCR reactions that include two or more target nucleic acids and may also include multiple primer sets in the same reaction mixture.

Applicants demonstrate that the use of a chemically-modified hot start DNA polymerase in combination with a volume exclusion agent, such as dextran or polyethylene glycol (PEG), significantly improves the coamplification of two or more target nucleic acids in a multiplex PCR reaction. As shown in the Examples section (discussed below), this is in comparison to the coamplification of two or more target nucleic acids in a PCR reaction with either a standard (non hot-start) DNA polymerase and a volume exclusion agent or hot start DNA polymerase and no volume exclusion agent.

Examples 1 and 2 of the specification, beginning on page 26, describe the coamplification of multiple target nucleic acids in the same PCR reaction, i.e., the PKC, SLP-65, ILGFMAR, c-fos, N-ras, fas, CD19 and CD5 murine genomic loci, all of which vary in size and sequence, using primers specific for each loci. Being genomic loci, the copy numbers of these targets were comparable. Amplification reactions were carried out with either (non hot-start) Taq DNA polymerase or a hot start DNA polymerase (HotStartTaq®) which, in this case, has been chemically-modified by reaction with an aldehyde and various concentrations (0%-6%) of either dextran or polyethylene glycol (PEG) as a volume exclusion agent.

As seen in the results shown in Table 2 on pages 28-29, the amplification reaction carried out with a chemically-modified hot start DNA polymerase in the presence of either 1%, 3%, or 6% dextran showed up to a 6-fold increase in PCR product yield as compared with hot start DNA polymerase with 0% dextran. In contrast, also as seen on page 29, the same reaction performed with standard Taq DNA polymerase yielded, for the most part, no detectable PCR product regardless of the concentration of dextran in the amplification reaction mixture.

Similar results are seen in Table 3 on page 30, where the same reactions described above were performed in the presence of either 1%, 3%, or 6% PEG. Similar to the results shown in Table 2, the presence of hot start DNA polymerase and PEG yielded up to a 12-fold increase in PCR product as compared to the amplification reaction containing hot start DNA polymerase and 0% PEG. Also, similar to the results shown in Table 2, the amplification reaction carried out in the presence of standard Taq DNA polymerase yielded almost no PCR product regardless of the concentration of PEG in the amplification reaction.

Therefore, the results of the present specification clearly demonstrate that a multiplex PCR coamplification reaction containing multiple target nucleic acids and multiple primers, each target nucleic acid being present in the reaction mixture at a concentration that is no more than 10-fold different than the concentration of any other target nucleic acid in the reaction, shows a significant improvement in yield of final product when the amplification reaction is performed in the presence of hot start DNA polymerase which has been chemically-modified and becomes active after incubation at temperatures above 50°C, in combination with from 1% to 6% of a volume exclusion agent, as compared to the reaction carried out in the presence of hot start DNA polymerase and 0% volume exclusion agent or, surprisingly, as compared to the same amplification reaction carried out in the presence of standard Taq DNA polymerase and regardless of the concentration of volume exclusion agent.

Applicants assert that the claims of the present application are clearly distinguishable from the teachings of the Backus reference. Backus discloses the use of a DNA polymerase inactivated with the TP4 monoclonal antibody (see, column 15, lines 1-5). As discussed above, the Examiner notes that the antibody is inactivated at higher temperatures. There is no teaching in Backus to combine a volume exclusion agent and a chemically-modified hot start polymerase for the coamplification of two or more target nucleic acids present at comparable, i.e., less than 10-fold different, copy numbers as disclosed in the present specification.

Examples 1-4 of Backus disclose a PCR reaction with a DNA polymerase inactivated by the TP4 monoclonal antibody wherein the reaction is comprised of one population of low copy nucleic acid (proviral HIV I DNA) and one population of high copy nucleic acid (human β-globin DNA) amplified in the presence of 10% PEG. As seen in Table I of Backus, the best level of PCR amplification in the presence of PEG 8000 only resulted in a 3.4-fold increase over control levels. (Compare, Example 1 of Backus, 0.1μM primer level, **4.25** dye signal, with Control A (0.1 μM primer level, **1.25** dye signal)). In contrast, the present application demonstrates that in a multiplex PCR reaction using hot start DNA polymerase which has been chemically-modified by reaction with an aldehyde group and becomes active after incubation at temperatures above 50°C, and the reaction having up to 8 different populations of nucleic acids, resulted in an over 4-fold increase in at least one of the populations with as little as 1% PEG and up to 12-fold increase in at least one population of target nucleic acids with only 6% PEG.

Therefore, Backus only discloses that an antibody-blocked DNA polymerase in combination with a very high (10%) concentration of PEG is suitable for use in a PCR reaction involving two unequal targets, but there is no teaching that the co-use of a volume exclusion agent in combination with a chemically-modified hot start DNA polymerase would lead to the unexpected results for coamplification of comparable targets shown in the Qiagen examples described above. In addition, Applicants note that the Backus patent never demonstrates that the disclosed method is suitable for coamplification of more than two target nucleic acids in a single reaction mixture.

With respect to the Bustin reference, this is a review article describing methods for quantifying mRNA gene transcripts by reverse transcription polymerase chain reaction (RT-PCR). According to Bustin, RT-PCR-specific errors in the quantification of mRNA transcripts are compounded by variation in the amount of starting material, particularly when samples are taken from different individuals. The section of Bustin referred to by the Examiner concerns the normalization, i.e., calibration, to account for the errors that occur as a result of significant variations in the amount of mRNA target transcripts in the starting material in RT-PCR reactions for quantifying mRNA transcripts again, particularly when comparing amplification of transcripts from samples taken from different individuals. According to Bustin, a common method for minimizing these quantification errors between samples is to include a cellular RNA in the reaction, one that is expressed at a constant level in various tissues and, if possible, expressed at the same level endogenously as the mRNA gene transcript under study. Therefore,

Bustin relates to normalization of an RT-PCR reaction using an RNA with known *in vivo* expression levels. Bustin does not teach or disclose the coamplification of two or more target nucleic acids in a reaction mixture with chemically-modified hot start DNA polymerase in combination with a polymeric volume exclusion agent where the target nucleic acids are present in comparable copy number in relation to the other target nucleic acids in the reaction mixture. As discussed above, Applicants have demonstrated that this improved method is suitable in a multiplex PCR reaction with multiple (in the present Examples, 8) target nucleic acids requiring 8 different primer sets. Bustin discloses the use of a known internal standard to set a benchmark for normalizing unknown variations between separate amplification reactions.

In fact, Bustin indicates that, at the time that review was published (2000), Applicants' method for the efficient amplification of multiple target nucleic acids in a reaction that included multiple sets of primers was virtually impossible to accomplish. According to Bustin,

> **"Multiplex RT-PCR . . .**
> The final difficulty [with multiplex RT-PCR] is associated with limitations caused by mutual interference of multiple sets of PCR primers, which can reduce the dynamic range of the sensitivity and make quantification unreliable. Furthermore, the efficiency of multiplex detection is significantly affected by the extension time and the concentrations of dNTPs, primers and $MgCl_2$ . . . Vastly different levels of target mRNAs will cause quantification problems even for real-time detection, as the exponential phase of amplification of the less abundant mRNA will not overlap with that of the highly abundant target." (See, Bustin, page 185, right column, 3rd paragraph.).

Finally, Bustin concludes,

> "Therefore, in practice, **if accurate quantification is the main aim**, it is probably best to limit multiplexing to the detection of two or three transcripts." (See, Bustin, page 185, right column, 3rd paragraph.) (emphasis added)

Therefore, Bustin discloses that (as of 2000) there existed significant limitations in the state of the art with respect to the efficiency of multiplex RT-PCR. As seen from above, the state of the art at the time the Bustin review was published was such that numerous obstacles existed to achieving reliable efficient amplification of nucleic acids in a multiplex PCR reaction, which obstacles went well beyond mere optimization of a reaction mixture but required the development of a novel approach concerning the interactive dynamics of all the components that were mixed together in one reaction. More importantly, Bustin never discloses or suggests any

solution to this problem other than to limit multiplexing reactions to two or three transcripts and no method is taught or suggested for even this "solution" to the problem. It is also noted that Bustin is primarily discussing multiplex RT-PCR reactions with "vastly different levels of target mRNAs" in the same reaction mixture and how impossible it was to achieve reliable quantitation. Therefore the Bustin reference, while it may identify a major problem existing in the field of multiplex RT-PCR in 2000, the best solution that is suggested for the problem is reducing the number of target nucleic acids in the reaction mixture. Bustin does not teach or suggest any aspect of Applicants' method for the amplification of two or more target nucleic acids present in comparable copy numbers with a chemically-modified hot start DNA polymerase in combination with a volume exclusion agent and, as such, the Bustin reference, in combination with Backus, Reed, and/or Demke does not make up for the lack of this teaching or suggestion in any of those references.

In view of the amendments herein and the foregoing remarks, reconsideration and allowance of Claims 1, 2, 4-16, 23, and 24 are respectfully requested.

35 U.S.C. §103

The Examiner has maintained the rejection of Claims 1-11 and 16 under 35 U.S.C. §103 as being unpatentable over Backus (*supra*), in view of Bustin (*supra*),

According to the Examiner,

> "Backus discloses a method of amplification of multiple target nucleic acids in the presence of a nonionic, polymeric volume exclusion agent (Abstract)." (See, Office Action, page 6.)

With respect to Bustin the Examiner states,

> "Bustin teaches an overview of the quantitation of mRNA using a variety of methods, including quantitative real-time RT-PCR, a method which incorporates a variety of means of detection, including hybridization probes (Abstract)." (See, Office Action, page 12.)

As stated above, Backus does not teach or suggest a method for the coamplification of two or more target nucleic acids present at comparable copy numbers wherein the reaction mixture includes a chemically-modified hot start DNA polymerase in combination with a volume exclusion agent. Backus teaches coamplification of a high-copy number and a low-copy number

target nucleic acid in a reaction mixture that includes an antibody inactivated DNA polymerase and a high concentration (10%) of PEG.

Bustin is a review article that suggests a method for calibrating an RT-PCR reaction for quantifying mRNA transcripts, where the reaction includes more than one target mRNA transcript which results in a starting reaction mixture having "vastly different levels of target mRNAs". More importantly, Bustin discusses major problems existing in the field of RT-PCR concerning the reliable quantitation of mRNA in a multiplex RT-PCR reaction, particularly when multiple primers are included, however Bustin never discloses or suggests any solution to the problem other than to reduce the number of target mRNAs in the reaction.

Applicants on the other hand have not only identified significant problems existing in the field for reliably and efficiently coamplifying multiple target nucleic acids in a single PCR multiplex reaction, Applicants also demonstrate a novel solution to overcome these problems, which is not taught or suggested in the cited art.

For the reasons set forth above, neither the Backus nor Bustin reference, alone or in combination, teach Applicants' improved method for the coamplification of two or more target nucleic acids.

Entry of the claim amendments, reconsideration and allowance of Claims 1, 2, 4-16, 23-25, are respectfully requested.

Respectfully submitted,

Leon R. Yankwich, Reg. No. 30,237
David G. O'Brien, Reg. No. 46,125
Attorneys for Applicants
YANKWICH & ASSOCIATES, P.C.
201 Broadway
Cambridge, Massachusetts 02139
telephone: 617-374-3700
telecopier: 617-374-0055

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Melanie A. McFadden
Mélanie A. McFadden